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14. ABSTRACT

The immune responses during lethal filovirus infection and the correlates of protective immunity in vaccinated macaques are not well understood. This study sought to develop assays that can predict protection with the various vaccine platforms designed to provide immunity to filovirus (Ebolavirus (EBOV) and Marburgvirus (MARV)) infection. A secondary aim was to better understand the aspects of virus on the immune response in animals that receive no intervention. As part of these efforts, we (Task 1) profiled the functional and phenotypic status of immune cells in Ebola virus (EBOV)-infected non-human primates and (Task 2) developed assays to assess virus-interactions with antigen presenting cells with the view that these interactions will influence the immune dysregulation that occurs during EBOV infection and sought to identify strategies to overcome virus-induced immune dysregulation. The latter work focused on macrophages and dendritic cells, which are important targets of Ebola viruses in vivo and are thought to mediate dysregulated immunity during infection in vivo.

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Ebola virus, filovirus, vaccine, dendritic cell responses

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INTRODUCTION: The immune responses during lethal filovirus infection and the correlates of protective immunity in vaccinated macaques are not well understood. This study aims to develop assays that can predict protection with the various vaccine platforms designed to provide immunity to filovirus (*Ebolavirus* (EBOV) and *Marburgvirus* (MARV)) infection. A secondary aim is to better understand the aspects of virus on the immune response in animals that receive no intervention. As part of these efforts, we **(Task 1)** profiled the functional and phenotypic status of immune cells in Ebola virus (EBOV)-infected non-human primates and **(Task 2)** developed assays to assess virus-interactions with antigen presenting cells with the view that these interactions will influence the immune dysregulation that occurs during EBOV infection and will identify strategies to overcome virus-induced immune dysregulation. The latter work focuses on macrophages and dendritic cells, which are important targets of Ebola viruses in vivo and are thought to mediate dysregulated immunity during infection in vivo (1-4).

Key Research Accomplishments and Reportable Outcomes

A. Assessment of immune cell numbers and phenotypes in EBOV-infected NHPs.

Background. EBOV infection is proposed to dysregulate immune responses and this likely contributes to failure of the host to clear the infection. It is therefore important to characterize the impact of in vivo EBOV infection on immune cell populations. Defining the impact of EBOV infection on host immune responses will allow investigators to assess how effective therapeutic or vaccine strategies mitigate immune dysregulation during the course of infection.

Therefore, EBOV-infected macaques were bled by USAMRIID investigators on days 1-8 post-infection, and the samples were characterized individually. Flow cytometry was performed by USAMRIID personnel. Basler lab personnel analyzed the data. Note that the volumes of blood which can be obtained from an individual animal depends upon its weight and are limited by animal use protocols. Therefore, not all animals are bled on all days.

Results.A.1. Peripheral CD4+ lymphocyte numbers were relatively constant, showing a modest increase between days 2-4 and a modest drop decline at day 8 (Figure 1).

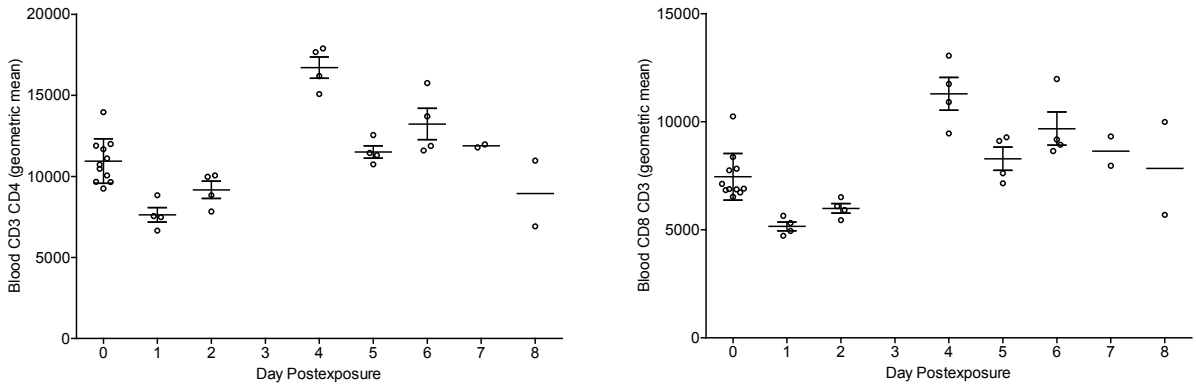

Figure 1: Peripheral CD4+ and CD8+ T-cell Numbers

Points indicate values for individual macaques; cells were stained with antibodies to T-cell markers CD3, CD8, and CD4 and analyzed by flow cytometry. Relative numbers of peripheral cells are charted over time. Mean is indicated (simple horizontal lines) if there are 2 or more data points per day; standard deviation is indicated if there are 3 or more data points per day.

A.2. We sought to determine which immune cells are infected *in vivo*. To do this, CD14+ (monocytes/macrophages), CD4+ T lymphocytes, CD8+ T lymphocytes and CD20+ (B lymphocytes) were stained with a monoclonal antibody (9C11) that recognizes the EBOV glycoprotein (GP). Consistent with published data, little to no GP+ T lymphocytes were detected in either blood (PBMCs) or spleen. CD14+ cells were very obviously infected and infected CD14+ cell numbers increase substantially in blood after day 6 and in spleen after day 7, consistent with uncontrolled, systemic virus replication in these cells. Surprisingly, we also detected with 9C11, B lymphocytes that are apparently GP-positive. This could reflect a previously unrecognized population of EBOV-infected lymphocytes or indicate that EBOV virions or even free GP may associate with these cells. The basis for these cells positive reaction with 9C11 deserves further investigation.

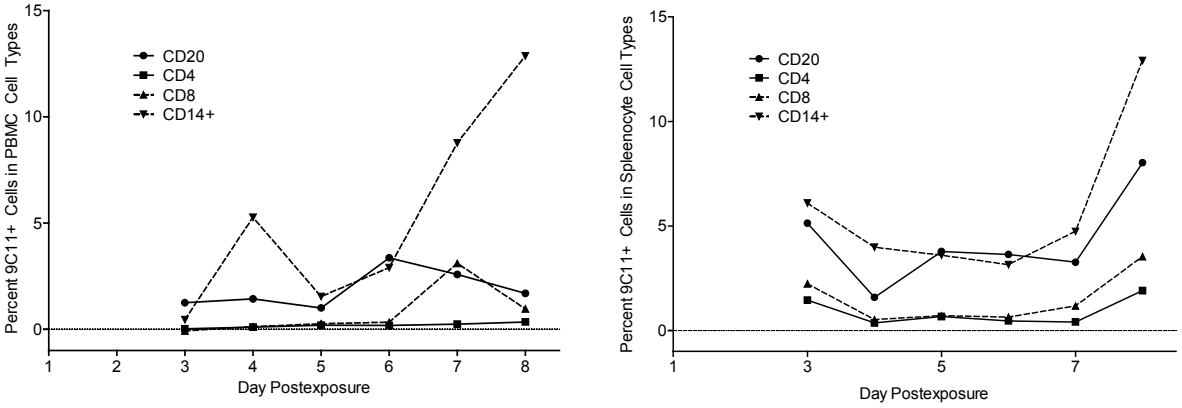

Figure 2: Percent Cell Types in 9C11+ (GP+) PBMC or Spleenocytes in Rhesus Macaques Exposed to Aerosolized ZEBOV

Points indicate values for individual macaques; to identify the percent 9C11+ (GP+) cells, lineage markers were first used to subdivide the PBMC or spleen cells. T lymphocytes CD3+CD20- were subdivided into CD4 and CD8 T cells (CD3+ CD4+ and CD3+ CD8+) populations while B cells were defined as CD3- CD20+ cells. The CD3-CD20- fraction was further gated for CD14+ myeloid cells. 9C11+ cells were defined as the percentage of specific cells (CD3+CD4+) that stained positive with anti-GP+ 9C11 antibody subtracted from control isotype antibody background.

A.3. To better characterize the "dysregulation" of host immunity, which may contribute to the inability of the host to control the infection, we defined the functional status of immune cells in infected animals.

A.3.1. Antigen presenting cells were examined ex vivo by flow cytometry for the surface markers CD11c and CD14 as well as HLA-DR and CD86. The monocytes/macrophages (CD14+ HLA-DR+ cells) (Figure 3) exhibit a different phenotype as compared to DCs (CD11c+ CD14- cells)(Figure 4).

Class II (HLA-DR+) and costimulatory marker upregulation in antigen presenting cells is thought to be required for proper T helper cell stimulation. Macrophages show some increase in HLA-DR and CD86 levels (Figure 4) later in infection, while in DCs CD86 and HLA-DR expression drops off precipitously by day 8. This latter effect may reflect dysfunction in the DCs late in infection (Figure 3).

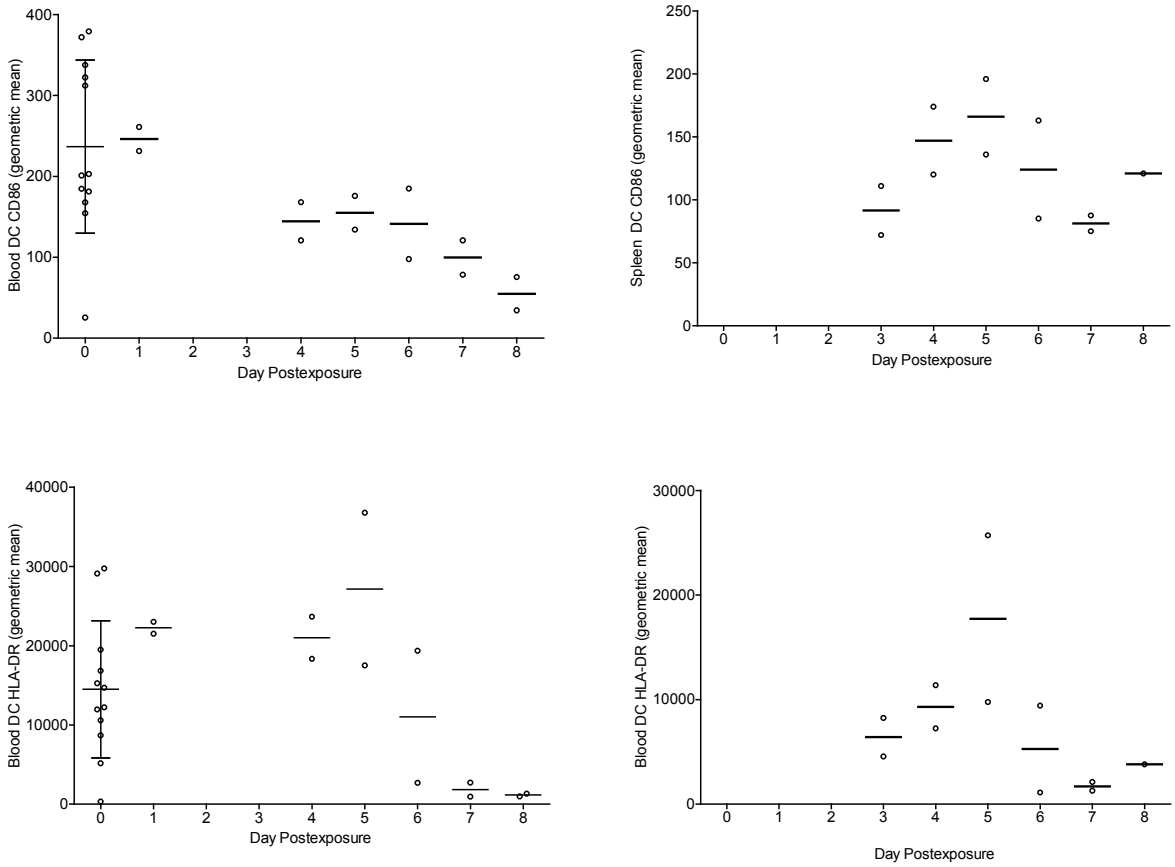

Figure 1: Phenotypes of Blood or Spleen CD11c+CD14- Dendritic Cells in Rhesus Macaques Exposed to Aerosolized ZEBOV

Points indicate values for individual macaques; cells; CD86 (top), HLA-DR (bottom); blood (left), spleen (right). Mean is indicated if there are 2 or more data points per day; standard deviation is indicated if there are 3 or more data points per day. Blood and spleen from *Ebolavirus*-infected NHPs were sampled at the indicated times. Cells were stained with antibodies to antigen-presenting cell markers as well as stimulation markers and analyzed with a flow cytometer.

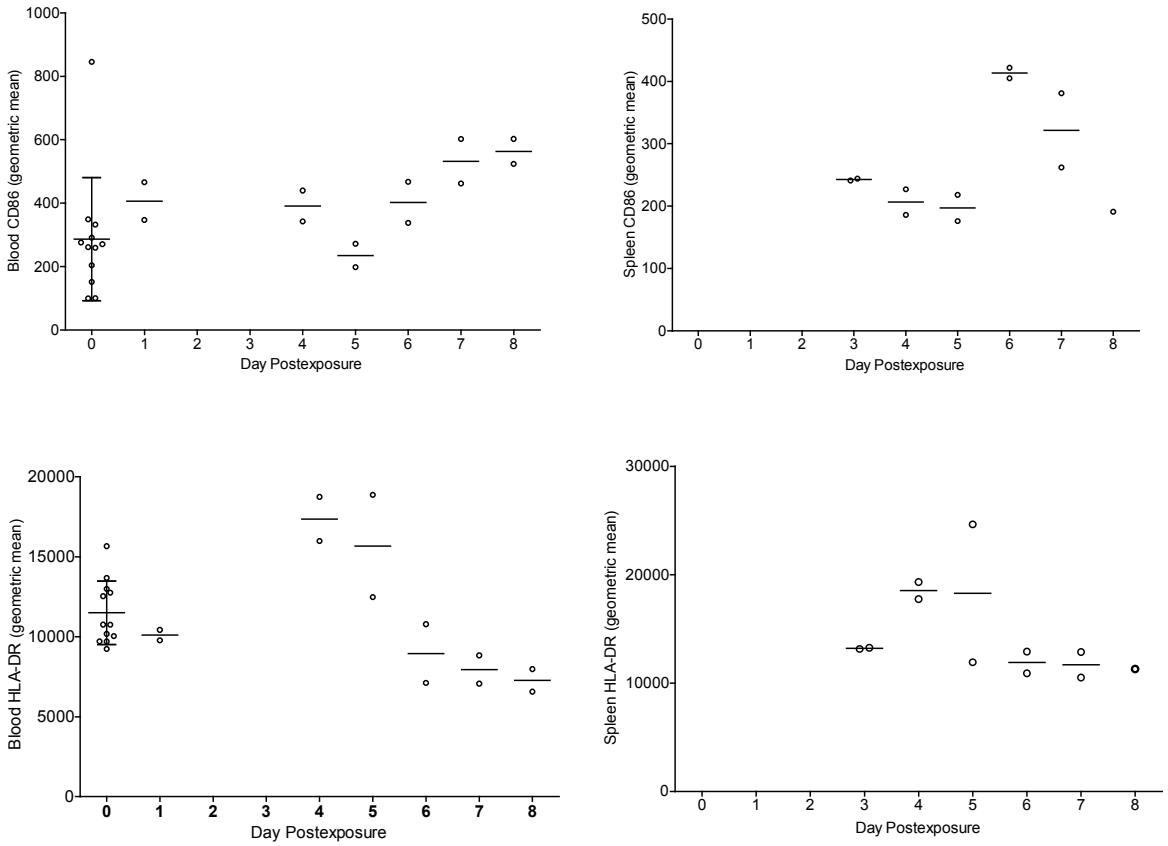

Figure 4 : Phenotypes of Blood or Spleen CD14+HLA-DR+ Myeloid Cells in Rhesus Macaques Exposed to Aerosolized ZEBOV (12 macaques, Iteration 1)

Points indicate values for individual macaques; CD86 (top), HLA-DR (bottom); blood (left), spleen (right). Mean is indicated if there are 2 or more data points per day; standard deviation is indicated if there are 3 or more data points per day. Blood and spleen from *Ebolavirus*-infected NHPs were sampled at the indicated times. Cells were stained with antibodies to antigen-presenting cell markers as well as stimulation markers and analyzed with a flow cytometer.

A.3.2. The activation status of CD4+ and CD8+ T lymphocytes was also examined in blood and spleen by examining levels of cell surface activation markers CD69 and CD25 for CD4+ cells and CD69 and CD62L for CD8+ cells. For CD4+ T cells, both activation markers increased substantially late in infection (days 6-8) (Figure 5). For CD8+ T cells, both CD25 and CD62L increase late in infection, most notably in the spleen (Figure 6).

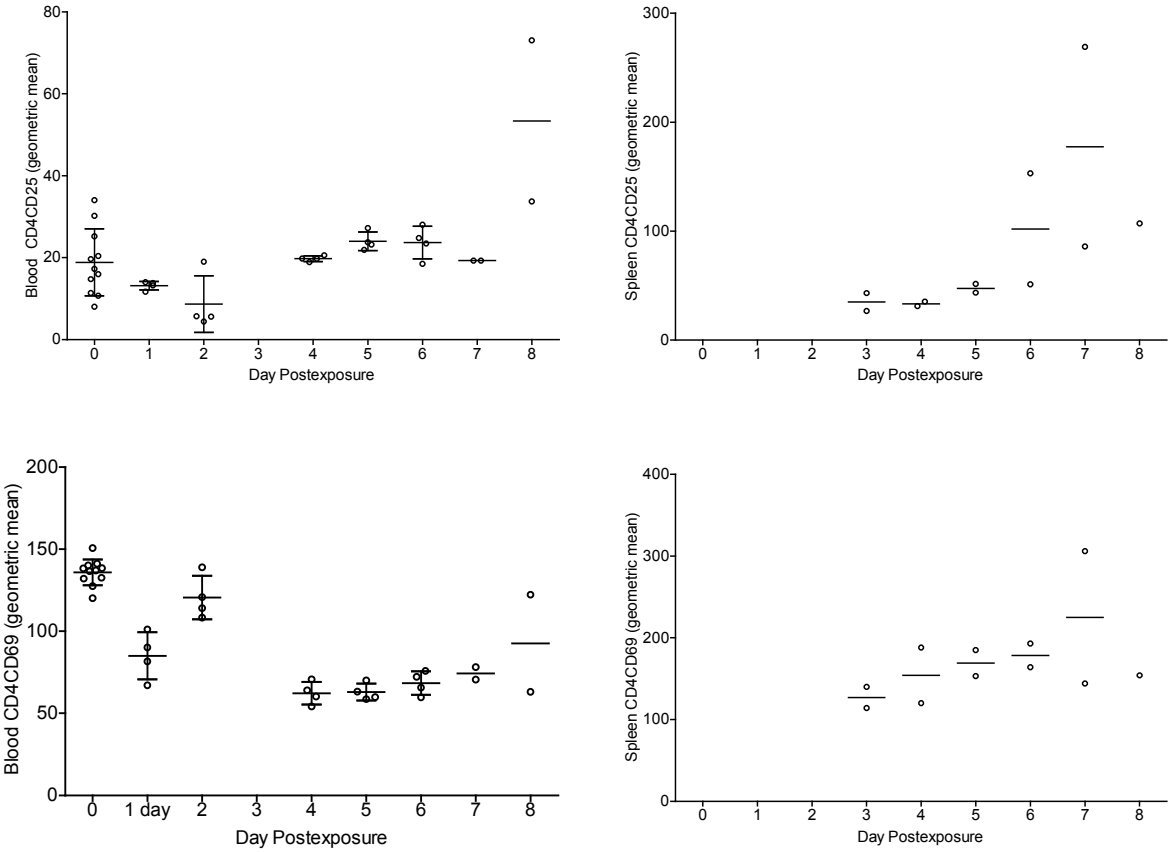

Figure 5: Phenotypes of Blood or Spleen CD3+CD4+ T cells in Rhesus Macaques Exposed to Aerosolized ZEBOV

Points indicate values for individual macaques; CD25 (top) and CD69 (bottom); blood (left), spleen (right). Mean is indicated if there 2 or more data points per day; standard deviation is indicated if there 3 or more data points per day. Blood and spleen from *Ebolavirus*-infected NHPs were sampled at the indicated times. Cells were stained with antibodies to antigen-presenting cell markers as well as stimulation markers and analyzed with a flow cytometer.

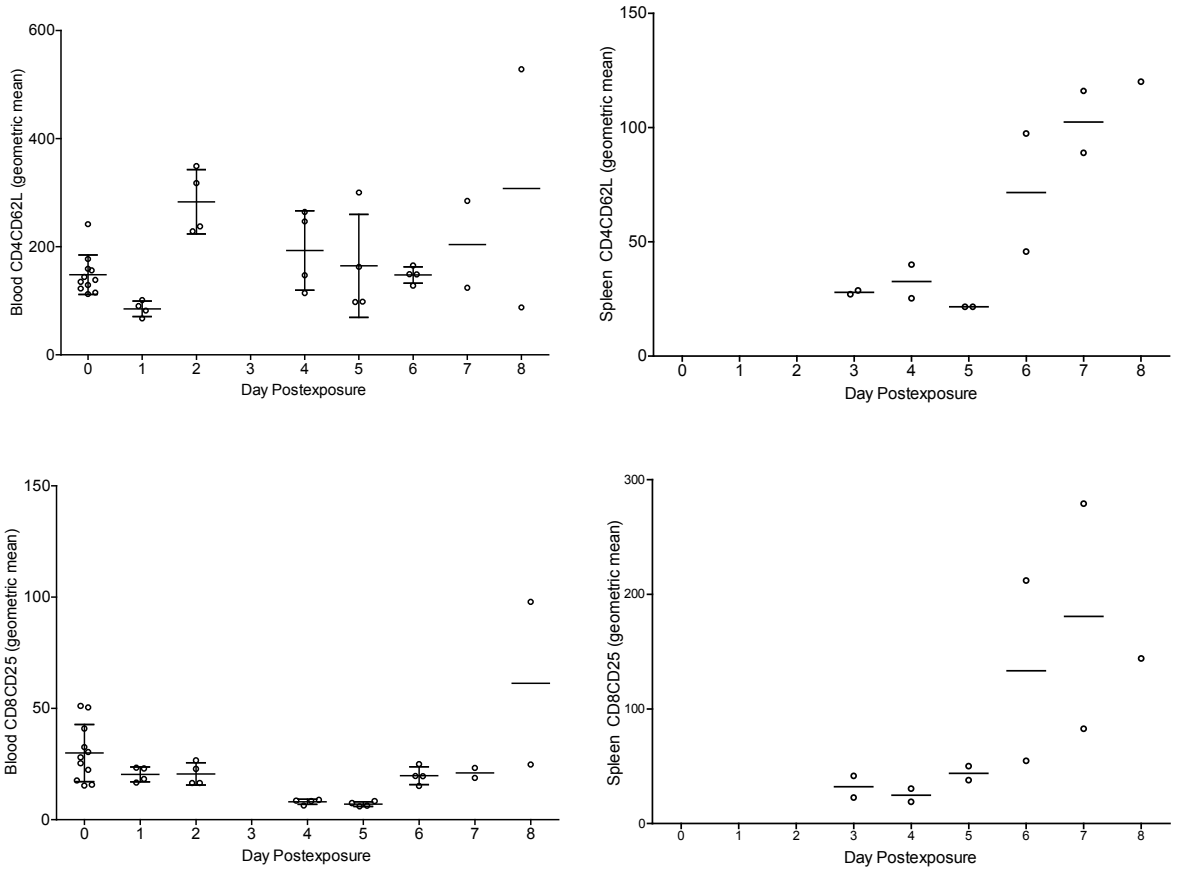

Figure 6: Phenotypes of Blood or Spleen CD3+CD4+ T cells of Rhesus Macaques Exposed to Aerosolized ZEBOV

Points indicate values for individual macaques; CD4+CD3+CD62L (top), CD8+CD3+CD25 (bottom); blood (left), spleen (right). Mean is indicated if there are 2 or more data points per day; standard deviation is indicated if there are 3 or more data points per day. Blood and spleen from *Ebolavirus*-infected NHPs were sampled at the indicated times. Cells were stained with antibodies to antigen-presenting cell markers as well as stimulation markers and analyzed with a flow cytometer.

B. Characterization of virus interactions with and entry into antigen presenting cells. As antigen presenting cells are important targets of EBOV infection in vivo (1-4), it is important to define the molecular basis for viral tropism towards these cells. Understanding the basis of viral tropism will suggest approaches to mitigate viral pathogenesis.

B.1. **Mutations in the Ebolavirus envelope glycoprotein restrict virus entry in a host species-specific manner.**

We studied entry of EBOV virus-like particles (EVLPs) as a surrogate for live virus, into murine peritoneal cells, because mouse-adapted Ebola viruses are lethal by this route but not by several other routes of infection. By identifying targets of virus entry and infection, we hope to gain insight into the connection between host cell tropism and virulence and to identify cells targeted by EVLPs to induce protective immune responses. We identified CD11b+ myeloid cells that include antigen-presenting cells (APC) as permissive for EBOV GP-mediated entry. Strikingly, however, a previously demonstrated entry-defective mutant, GP-F88A, entered

mouse, but not human APCs. Like wild-type (wt) GP, GP-F88A-mediated entry is cathepsin-dependent, likely proceeds via macropinocytosis and requires a fusion event but does not require the GP mucin-like domain. F88 occupies the same hydrophobic region as L111, I113, L122 and F225. Mutation of these residues resulted in preferential entry into several cell types, but not in a species-specific manner. However, GP triple-mutant K114A, K115A, K140A, previously shown to impair receptor binding in primate cells, is able to mediate entry into murine but not human APCs. These data demonstrate a role for GP residues F88, K114, K115 and K140 in EBOV host range restriction and suggest that EBOV may enter human and mouse cells by different mechanisms. This study has been submitted to Journal of Viroolgy and is currently under review.

Entry of Ebola virus into monocytes occurs during their differentiation

Zaire Ebola virus (EBOV) is reported to productively infect monocytes as well as macrophages and dendritic cells (DCs). However, other studies demonstrate that the EBOV glycoprotein (GP) can mediate entry into macrophages and DCs but not into undifferentiated primary human monocytes or THP-1 monocytes. We used an Ebola virus-like particle (VLP)-based entry assay and confirmed that EBOV entry into primary human or mouse monocytes is severely restricted as compared to macrophages and DCs, while entry mediated by vesicular stomatitis virus glycoprotein (VSV G) is efficient. Differentiation of monocytes into macrophages and DCs by any of several stimuli renders them permissive for entry. Plated monocytes slowly undergo spontaneous differentiation. VLP entry kinetics studies demonstrate EBOV entry into fully differentiated DCs occurs within four hours post-infection, while entry into plated monocytes requires twelve hours. Similarly, EBOV infection is delayed in plated monocytes relative to DCs as determined by onset of viral nucleoprotein mRNA transcription. Furthermore, the differentiation status of these APCs affects the magnitude and cytokine response profile to EBOV infection. Microscopy studies demonstrate that VLPs attach to the surface of undifferentiated monocytes and remain bound until a significant number of particles are endocytosed. Quantitative RT-PCR of freshly isolated monocytes demonstrates higher expression levels of EBOV entry restriction factors interferon induced transmembrane protein (IFITM) 1, 2 and 3 as compared to DCs. In contrast, cathespin B and NPC1, essential EBOV entry factors, are substantially upregulated in DCs versus monocytes. Finally, NPC1 overexpression in undifferentiated THP-1 monocytes partly rescues EBOV entry. These data suggest that EBOV can initiate the viral entry process in monocytes. However, complete entry is associated with monocyte differentiation where expression levels of IFITM are downregulated and NPC1 and other essential entry factors are upregulated. These data provide new insights into how EBOV initiates infection of cell types critical to the outcome of infection in vivo. We are currently preparing a manuscript describing these data for submission.

Conclusions. As outlined above, our studies for Task 1 hgave defined the impact of *in vivo* EBOV infection on immune cell populations. Defining the impact of EBOV infection on host immune responses will allow investigators to assess how effective therapeutic or vaccine strategies mitigate immune dysregulation during the course of infection.

Our studies in Task 2 have provided new insight into the interactions between the EBOV GP and antigen presenting cells, cells thought to be critical for the outcome of filovirus infection in vivo.

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APPENDICES: None